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Cryptosporidium and host resistance: historical perspective and some novel approaches

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Abstract

Cryptosporidium parvum is recognized as a major cause of diarrheal disease in neonatal bovine calves. In addition, this protozoan parasite has emerged as an important cause of disease in both immunocompromised and immunocompetent humans. Despite years of research, no consistently effective means of prevention or treatment are readily available for cryptosporidiosis in any species. Infection through ingestion of contaminated water has been widely documented; *C. parvum* was reported to be responsible for the largest waterborne outbreak of infectious disease in US history. In addition to its role as a primary disease agent, *C. parvum* has potential to initiate or exacerbate other gastrointestinal disorders, such as inflammatory bowel disease. Thus, control of *C. parvum* infection in both animals and humans remains an important objective. Research in our laboratory has focused on understanding mechanisms of resistance to *C. parvum*. We have demonstrated that acquisition of intestinal flora increases resistance to *C. parvum*. Substances present in the intestinal mucosa of adult animals can transfer resistance when fed to susceptible infants. Both expression of intestinal enzymes and rate of proliferation of epithelial cells may be altered following *C. parvum* infection. These and other changes may have profound effects on host resistance to *C. parvum*.

Keywords: *Cryptosporidium parvum*, diarrheal disease, innate immunity, host resistance, intestinal flora

Introduction

In this review I will present a brief history of *Cryptosporidium parvum*, highlighting what is known of the host immune response to the parasite, and the paucity of effective drug treatments or vaccines against infection. This information is provided for background; excellent reviews are available that explore these topics in more depth (Fayer *et al.*, 2000; Chen *et al.*, 2002; Riggs, 2002). In the remainder of the paper, I will summarize both published and unpublished studies from my laboratory that were designed to explore mechanisms of resistance to *C. parvum* that do not seem to be related directly to a specific acquired immune response. We believe that understanding the innate or age-related resistance to *C. parvum* infection (best exemplified by rodent models) may lead to better understanding of *C. parvum* infection in other species. Among the puzzles still unsolved: why are bovine calves so commonly infected with *C. parvum* and why do their resistant mothers fail to transmit effective immunity to infection? Why do humans appear to be one of the few species that have a highly susceptible adult population, and also, in some cases, seem susceptible to reinfection? The answers to these and other questions may prove to be found in host-response strategies that complement specific acquired immunity.

A brief history of *C. parvum*

C. parvum is an intestinal protozoan parasite that causes enteric infection and diarrhea in many species of mammals (O'Donoghue, 1995; Fayer *et al.*, 1997, 2000). *C. parvum* infection in calves has become a major eco-

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nomic concern for the producer. In a nationwide survey of 7369 preweaning dairy calves on 1103 farms, *C. parvum* was found in 22.4% of the calves. At least one calf tested positive on 59.1% of the farms tested. The frequency of infection was highest in calves aged 1–3 weeks. Forty-eight percent of the calves in this age group were positive for *C. parvum* (Garber *et al.*, 1994). *C. parvum* can cause diarrheal disease in calves in the absence of any other pathogens (Heine *et al.*, 1984b). Symptoms of cryptosporidiosis in calves include loss of appetite, weakness, depression and profuse watery diarrhea. Upon necropsy, intestines from infected calves show inflammation of intestinal epithelium, crypt hyperplasia and destruction of intestinal villi (Heine *et al.*, 1984b). In addition to its role as a primary pathogen, *C. parvum* is frequently found in association with other organisms known to cause calf diarrhea, such as *Escherichia coli*, rotavirus and coronavirus (Moon *et al.*, 1978). It is likely that destruction of the intestinal epithelium by cryptosporidia increases the susceptibility of young calves to infection with other enteric pathogens (Casey, 1991).

Recently, much attention has been directed to *C. parvum* as a cause of human disease. In immunocompetent humans infected with *C. parvum*, symptoms consist primarily of diarrhea, abdominal pain and, less frequently, vomiting. Disease is usually self-limiting, resolving in a few weeks. In contrast, in immunocompromised hosts, such as patients with acquired immune deficiency syndrome (AIDS), *C. parvum* infection can be a life-threatening disease (reviewed by Kosek *et al.*, 2001; Hunter and Nichols, 2002). Diarrhea may be chronic and unremitting, leading to dehydration and severe weight loss. Parasite infestation in immunocompetent hosts is primarily in the distal small intestine; however, in immunocompromised hosts the entire gastrointestinal (GI) tract may be affected (Kosek *et al.*, 2001). In AIDS patients, parasites frequently lodge in the bile ducts, making the organisms extremely difficult to eradicate. There is also evidence that *C. parvum* infection may trigger or exacerbate inflammatory bowel disease (IBD). Following a massive outbreak of cryptosporidiosis in Milwaukee, a number of patients with quiescent IBD progressed to active disease (Manthey *et al.*, 1997). Additionally, *C. parvum* DNA was found in a low percentage of mucosal biopsies from patients with IBD (Chen *et al.*, 2001). In an animal model, *C. parvum* alone is sufficient to trigger the onset of IBD-like syndrome (Sonea *et al.*, 2002).

Outbreaks of cryptosporidiosis caused by ingestion of oocyst-contaminated water have been frequent and well documented (reviewed by Fayer *et al.*, 2000). In the most highly publicized outbreak, over 400 000 people were reported to be infected by drinking water in Milwaukee, Wisconsin in 1993 (MacKenzie *et al.*, 1994, 1995). Water quality monitored at the treatment plants remained within guidelines before and during the outbreak, although turbidity was increased, consistent with spring runoff into Lake Michigan, the source of the city water supply. Initial speculation as to the source of contamination included cattle along the rivers flowing into the lake, slaughterhouses and human sewage (Edwards, 1993). Subsequent to this outbreak, examination of genetic polymorphisms has revealed at least two genotypes of *C. parvum* (reviewed by Okhuysen and Chappell, 2002). Type 1 (recently named *Cryptosporidium hominis*, see Morgan-Ryan *et al.*, 2002) is found almost exclusively in humans, and is thought to be responsible for anthroponotic transmission. Type 2 is able to infect humans as well as cattle, rodents and possibly other species. This type is thought to be responsible for zoonotic transmission of cryptosporidiosis (Kosek *et al.*, 2001). Four isolates were typed from the Milwaukee outbreak and proved to be of the type 1 genotype, suggesting that human sewage was responsible for the outbreak (Peng *et al.*, 1997). However, the possibility of more than one source remains, given that only four samples were tested. While most waterborne outbreaks of human disease in the US for which genotyping was done revealed *C. parvum* type 1 as the probable source of infection, several large outbreaks in Europe and elsewhere have identified type 2, implicating livestock in the zoonotic transmission of cryptosporidiosis (Fayer *et al.*, 2000). Thus, there remains a great need to identify means of controlling *C. parvum* infection in calves and other species, not only to reduce the economic impact on the producer, but also to alleviate environmental and public health concerns.

Recent studies indicate that genetic recombination occurs in *C. parvum*. Two varieties of the type 2 strain were shown to recombine in infected mice to produce a hybrid variant (Feng *et al.*, 2002). These experimental findings raise the question of whether recombination occurs naturally and whether type 1 and 2 genotypes can recombine. Also of interest is the recent suggestion that several type 2 isolates may vary in virulence for humans, as suggested by dose–response studies (Teunis *et al.*, 2002). While *C. parvum* is the species most commonly associated with human disease, both immunocompetent and immunocompromised persons may be infected with other species, including *C. felis*, *C. meleagridis* and a dog genotype of *C. parvum* (Xiao *et al.*, 2001; Caccio *et al.*, 2002; Chalmers *et al.*, 2002; Ong *et al.*, 2002).

A major problem in controlling *C. parvum* is the lack of effective means for preventing or treating infection. A large number of antimicrobials have been tested for efficacy (reviewed by Blagburn and Soave, 1997). Many of these have shown some *in vitro* efficacy, i.e. ability to block infection of cultured epithelial cells. However, these results have not been replicated in controlled *in vivo* trials. Several classes of compounds, including macrolides, aminoglycosides and ionophores, have been

tested against *Cryptosporidium* in AIDS patients, with mixed success (Hunter and Nichols, 2002). One initially promising drug, paromomycin, was recently demonstrated to be no more effective than placebo in a controlled trial (Hewitt *et al.*, 2000). A number of compounds are currently under investigation (Moore *et al.*, 2001; Nelson and Rosowsky, 2001; Gilles and Hoffman, 2002). The most effective means of controlling infection in immunocompromised humans is to alleviate the underlying immunodeficiency. Treatment with anti-retroviral agents or protease inhibitors, and the subsequent increase in $CD4^+$ T-lymphocyte counts, has been successful in alleviating cryptosporidiosis in AIDS patients (Hunter and Nichols, 2002).

There are few controlled studies of drug therapy against cryptosporidiosis in farm animals. Most agents tested have proven to be ineffective or toxic (Blagburn and Soave, 1997). Paromomycin has been reported to be efficacious in a controlled study in calves, but it is not currently licensed for use in food animals (Fayer and Ellis, 1993). In addition to the questionable value of paromomycin in human cryptosporidiosis mentioned above, the high cost of this drug makes it unlikely that it will be of practical value in veterinary applications. Numerous reports of success in treating bovine cryptosporidiosis with off-label remedies, such as decoquinate, may be found in veterinary discussion forums on the internet. There is no evidence in the scientific literature to support these claims (Lindsay *et al.*, 2000). It is likely that a remarkable array of compounds will result in decreased infectious disease problems when coupled with improved management and hygiene (Harp and Goff, 1998). Once calves become infected, the most effective treatment of cryptosporidiosis is supportive therapy, i.e. electrolytes to maintain fluid balance during bouts of diarrhea.

Specific immunity to *C. parvum*

Much remains to be learned about the mechanisms by which specific immunity protects against *C. parvum* infection. Infected animals produce systemic and local antibody to parasite antigens, but there is no clear correlation between the presence of antibody and protection from disease (Whitmire and Harp, 1991; Peeters *et al.*, 1992). Efforts have been made to generate immune responses to *C. parvum* through vaccination with either whole-cell antigens or recombinant *C. parvum* proteins. These studies involve vaccination of pregnant animals, with the goal of protecting neonates through passive transfer of antibody or other factors in colostrum and milk. Transfer of relatively low-titered colostral antibody from dams to calves did not protect against infection (Harp *et al.*, 1989); however, colostrums with higher titers of antibody against *C. parvum* protected neonates against experimental challenge (Fayer *et al.*, 1989; Perryman *et al.*, 1999; Sagodira *et al.*, 1999). Due to the transient nature of colostral antibody in the gut (Butler, 1999), it is not clear how effective these regimens would be under field conditions, when calves are continuously exposed to virulent *C. parvum* in the first weeks and months of life.

Studies of immunity against other intracellular parasites have shown that cell-mediated immune responses are an important component of resistance. Data from studies in mice suggest that intact cellular immunity is critical for resistance to, and recovery from, *C. parvum* (reviewed by McDonald and Bancroft, 1998; Theodos, 1998). Immunocompetent mice clear *C. parvum* infection at about 3 weeks of age, but various strains of T-cell-deficient mice are unable to clear the infection, and remain persistently infected as adults (Heine *et al.*, 1984a; Ungar *et al.*, 1990; Harp *et al.*, 1992; Waters and Harp, 1996). Several studies have established a role for interferon-γ (IFN-γ) in both resistance to and recovery from *C. parvum* infection (Ungar *et al.*, 1991; Chen *et al.*, 1993a, b). Similarly, studies of the cell-mediated immune response of calves to *C. parvum* indicate that a cellular response is present to parasite antigens (Whitmire and Harp, 1991; Pasquali *et al.*, 1997; Wyatt *et al.*, 1997, 1999, 2001; de Graaf *et al.*, 1998; Fayer *et al.*, 1998).

Vaccination of newborn animals to stimulate an active immune response is not well studied. We have demonstrated that vaccination of newborn calves with killed *C. parvum* protected against experimental challenge at 1 week of age (Harp and Goff, 1995). In a field trial, this vaccine was ineffective, probably because calves were exposed to virulent *C. parvum* within the first days of life, before the vaccine was able to stimulate a protective response (Harp *et al.*, 1996). It seems likely that a successful strategy to protect newborn calves from *C. parvum* will include stimulation of a non-specific response in the first days of life, thus allowing time for the generation of specific immunity to the parasite.

Role of intestinal flora in resistance to *C. parvum*

Cryptosporidiosis is most prevalent in the young animals of susceptible species (reviewed by Casemore *et al.*, 1997). While *C. parvum* diarrhea is widespread in young calves, it is virtually never seen in adult cattle, or even in calves older than about 1 month (Angus, 1990; Casey, 1991). It is possible that changes in gut microflora, maturation of the intestinal epithelium or other age-related changes increase the resistance of older calves and adult cattle to *C. parvum* infection (Akili and Harp, 2000). Other studies have shown that, in addition to age-related changes, exposure to the parasite results in an immune response and subsequent resistance to reinfection (Harp *et al.*, 1990; Whitmire and Harp, 1991).

We have used mice as a model to study the role of

intestinal microflora in resistance to *C. parvum*. Adult mice are highly resistant to infection, even in the absence of any previous exposure to *C. parvum* (Sherwood *et al.*, 1982; Harp *et al.*, 1988; Harp, 1996). This age-related resistance begins at about 3 weeks of age, coincident with the acquisition of mature intestinal flora (Savage *et al.*, 1968; Davis *et al.*, 1973). The following paragraphs summarize previously published studies designed to define the role of intestinal flora in resistance of adult mice to *C. parvum*.

Germ-free and conventional flora-bearing adult mice were orally challenged with *C. parvum* oocysts. Mice were killed 1 week after challenge and were examined for evidence of *C. parvum* infection. *C. parvum* was found in either colon content or intestinal tissue of all 29 adult germ-free mice. In contrast, *C. parvum* was not seen in colon contents of any conventional adult mice, and was seen in intestinal sections of only 5 of 31 mice (Harp *et al.*, 1988). These results suggested several possibilities. The physical presence of bacteria might block receptor sites for the parasite, or the bacteria might produce substances toxic to *C. parvum*. Alternatively, the presence of bacteria in the intestine might trigger some component of the host immune system that increases resistance to *C. parvum* infection.

We then performed the following experiments. Conventionally reared adult mice, and germ-free adult mice as controls, were treated with antibiotics to eliminate culturable bacteria from their gastrointestinal tract. All mice were then challenged with *C. parvum* and killed 1 week later. *C. parvum* was found in 7 of 9 germ-free mice, but in only 4 of 12 conventional mice (Harp *et al.*, 1988). Thus, antibiotic-treated conventional mice, although free of culturable bacteria, remained more resistant to colonization with *C. parvum* than did antibiotic-treated, germ-free mice. These results support the hypothesis that the presence of bacteria in the intestine is not the key component of resistance. These results also confirm that the antibiotics used in the study had no direct effect on *C. parvum*, since the antibiotic-treated, germ-free mice remained susceptible.

These results suggest the possibility that the association of bacteria with the intestinal immune system provides a priming signal that results in enhanced resistance to *C. parvum* infection. This resistance persists even when the flora are removed. Our studies do not exclude the possibility that intestinal flora not culturable by our methods persisted in the antibiotic-treated mice. However, in view of the increased susceptibility of mice to other pathogens following similar antibiotic treatment (Kennedy and Volz, 1983, 1985; Que and Hentges, 1985; Ekenna and Scheretz, 1987), and the documented contribution of intestinal flora to development of the immune system (Olson and Wostmann, 1966; Gordon and Pesti, 1971), a role for flora in priming the immune system against *C. parvum* infection seems likely.

To further examine the relationship between intestinal flora and resistance to *C. parvum*, we studied infection in severe combined immunodeficient (SCID) mice, which lack both T- and B-lymphocyte subsets, due to a genetic defect in rearrangement of receptor genes (Bosma *et al.*, 1983). These mice had been reported previously to be susceptible to chronic *C. parvum* infection as adults (Mead *et al.*, 1991). By assessing the effects of intestinal flora in a host unable to generate a specific immune response against *C. parvum*, we sought to further define the relative importance of age-related and specific immunity. We obtained CB-17/IcrTac-scid (SCID) and CB-17/IcrTac (control, immunologically normal) flora-bearing mice, as well as germ-free SCID mice. In our initial studies, we confirmed that the flora-bearing SCID mice became chronically infected with *C. parvum*, while the immunologically normal control mice did not (Harp *et al.*, 1992). However, the SCID mice did not begin to show colonization with *C. parvum* until 5–7 weeks after challenge (Harp *et al.*, 1992). We were struck by the ability of these severely immunocompromised mice to resist infection for several weeks following exposure. To determine whether intestinal flora played a role in this early resistance to *C. parvum*, we challenged both flora-bearing and germ-free SCID mice with *C. parvum*, and examined intestinal tissues for signs of infection. Germ-free SCID mice were heavily infected with *C. parvum* 3 weeks after challenge, while no parasites were found in intestines of flora-bearing SCID mice 3 weeks after challenge (Harp *et al.*, 1992). These data indicated that intestinal flora contributed to resistance of adult mice to *C. parvum* infection, even in the absence of a specific immune response.

Previous studies have indicated that IFN-γ is a key mediator in resistance to, and recovery from, *C. parvum* infection (Ungar *et al.*, 1991; Chen *et al.*, 1993a, b). It is also known that SCID mice are able to produce IFN-γ through a non-T-cell pathway, and this pathway has been shown to be important in the resistance of SCID mice to other intracellular pathogens (Bancroft *et al.*, 1991). Production of IFN-γ can be stimulated by the presence of an intestinal flora (Wherry *et al.*, 1991). In sum, these studies suggest that increased susceptibility to *C. parvum* seen in germ-free adult mice (and neonatal mice before the acquisition of intestinal flora) may be due, at least in part, to deficiencies in IFN-γ production.

To further examine this possibility, we studied the effects of colonization with lactic-acid producing bacteria (Lb) on susceptibility of immunodeficient adult mice to *C. parvum*. Studies in mice have shown that colonization of the intestine with Lb can have beneficial effects, including increased resistance to pathogens (De Simone *et al.*, 1993, 1995). Several studies have shown that dosing of mice with Lb resulted in reduced colonization following challenge with *C. parvum* (De Simone *et al.*, 1995; Alak *et al.*, 1997). *Lactobacillus reuteri* was shown to diminish infection with *C. parvum* in mice rendered immunodeficient by a murine retrovirus (Alak *et al.*,

1997). It is thought that *L. reuteri* inhibits infection through production of an antimicrobial substance, reuterin (Casas and Dobrogosz, 1997). We examined the effects of *L. reuteri* on *C. parvum* infection in T-cell receptor (TCR)α-deficient mice. These mice lack αß T lymphocytes and become chronically infected with *C. parvum* when exposed as either neonates or adults (Waters and Harp, 1996). Subsequent to infection, they show rapid onset of inflammatory bowel disease, which we have shown can be triggered by *C. parvum* in the absence of any other intestinal flora (Sacco *et al.*, 1998). We found that colonization with *L. reuteri* lessened the severity of *C. parvum* infection, and reduced the degree of inflammation due to IBD (Waters *et al.*, 1999).

In order to examine mechanisms by which Lb might reduce *C. parvum* infection, we performed further studies with germ-free SCID mice. Four groups of mice were housed in separate isolators. The first and fourth groups received five oral doses of phosphate-buffered saline (PBS) every other day for 10 days. The second and third groups received five doses of Lb. Bacterial preparations consisted of 10^{11} viable bacteria per dose and contained a mixture of *Streptococcus*, *Bifidobacterium* and *Lactobacillus* spp. (Harp, 1996). One week after completion of dosing, fecal pellets were cultured to confirm colonization of mice in groups 2 and 3 with Lb. Mice in groups 1 and 2 were then challenged with *C. parvum*. Three weeks later, all mice were killed. One half of the ileum, cecum and colon was fixed in formalin and processed for histological examination for *C. parvum* infection. Tissues were scored as 0, no *C. parvum* found; 1, few *C. parvum* found; 2, many *C. parvum* found. The mucosa was stripped from the other half and processed for mRNA extraction (Chomczynski and Sacchi, 1987). Gene transcript levels were measured by reverse transcriptase polymerase chain reaction (PCR), as described previously (Havell and Rogerson, 1993). Germ-free SCID mice challenged with *C. parvum* were more heavily infected than were SCID mice colonized with Lb (ileal/cecal score of 1.7 versus 0.8, and colon score of 0.3 versus 0.0, respectively). No *C. parvum* was found in the control groups 3 and 4 (Harp, 1996). PCR products for IFN-γ mRNA were seen for each individual mouse in groups 1 and 2, and no bands were seen from any mice in groups 3 and 4 (Fig. 1). Thus, colonization of the intestine with Lb appeared to reduce infection with *C. parvum*. However, there was not a correlation between colonization with Lb and expression of IFN-γ mRNA in intestinal tissues. Rather, those mice challenged with *C. parvum* expressed IFN-γ mRNA, regardless of whether they were colonized with Lb. In addition, mice colonized with Lb alone did not express measurable IFN-γ mRNA, at the time point assayed in these studies.

These results suggest that colonization of the mice with Lb may be protecting them from *C. parvum* infection by a mechanism not directly involving IFN-γ. Alternatively, IFN-γ may have been induced early in the mice receiving Lb alone, and disappeared by the time of necropsy. In the mice that received both Lb and *C. parvum*, the additional stimulation may have resulted in increased levels of IFN-γ that were measurable at the time of necropsy. It has been reported that interleukin-12 induced a transient increase in IFN-γ mRNA, while *C. parvum* induced a longer-lasting increase (Urban *et al.*, 1996). It is possible that Lb might provide a non-specific stimulus for IFN-γ production prior to *C. parvum* infection, which is able to modulate the immune response and lessen the severity of infection. Further study would be required to distinguish among these possibilities. In sum, although the mechanism remains undefined, these studies clearly indicate that intestinal microflora are able to mediate resistance to *C. parvum* infection in both immunocompetent and immunocompromised mice.

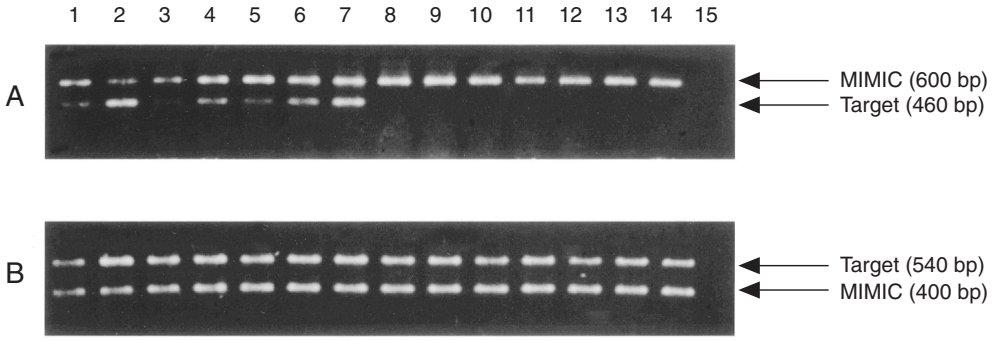

Fig. 1. Agarose gel (1.4%) of PCR products stained with ethidium bromide. (A) IFN-γ cDNA from individual samples from mice in the four groups (bottom bands) and corresponding IFN-γ MIMIC (top bands). Lanes 1–3, PBS + *C. parvum*; 4–7, Lb + *C. parvum*; 8–11, Lb alone; 12–14, PBS alone; and 15, control, no RNA template. (B) β-Actin cDNA from individual samples from mice in the four groups (top bands) and corresponding β-actin MIMIC (bottom bands). Lanes are the same as for (A). (Reprinted with permission from Harp, *Journal of Immunology and Immunopharmacology* **16**: 11–16, 1996.)

Resistance factors in intestinal mucosa

The experiments described above demonstrated that immunocompetent adult mice remained resistant to *C. parvum* infection even after depletion of existing intestinal microflora. In addition to possible effects on stimulation of the immune response, these data suggest that a physiological change that accompanies maturation of the intestinal mucosa may contribute to the resistance of adult animals to *C. parvum*. Thus, we hypothesized that a protective factor might be present in adult intestine that could transfer resistance to an otherwise susceptible infant. To test this hypothesis, we scraped intestinal mucosa from the ilea of adult rats (not previously exposed, but innately resistant to *C. parvum* infection), suspended it in PBS, and fed it to infant rats (normally susceptible to *C. parvum* infection). Rats received mucosal preparations starting at 3 days of age and continuing until 14 days of age. Control rats received PBS alone, or were not treated. All rats were challenged with *C. parvum* at 9 days of age. Rats were necropsied at 15 days of age and assessed for evidence of *C. parvum* infection by examination of colon content, and histological examination of ileal tissue for parasites. We found that there was a significant reduction in both the percent of rat pups with oocysts present in feces, and with parasites present in ileal tissue in the group receiving mucosal preparations compared with controls (Akili and Harp, 2000). Interestingly, pups in the group receiving mucosal preparations that did become infected with *C. parvum*, had significantly fewer oocysts in feces compared with numbers of oocysts seen in the feces of pups not receiving mucosal preparations. Thus, the mucosal preparation reduced both numbers of animals infected, and the severity of infection in animals not fully protected from infection (Akili and Harp, 2000). To determine if the activity of the preparation was species specific, we treated infant rats with ileal mucosal preparations from bovine calves that had been exposed to *C. parvum* previously and recovered from infection. This preparation had no effect on the susceptibility of the infant rats to *C. parvum* infection. However, when infant rats were treated with mucosal preparations from adult cows, they were protected to a similar degree as was the group that received adult rat mucosal preparations (Akili and Harp, 2000). Based on previous studies, calves that recover from *C. parvum* infection are resistant to reinfection (Harp *et al.*, 1990). Thus, it would appear that even though the calves donating the mucosal preparations in this study were no longer susceptible to infection with *C. parvum* themselves, they had not yet developed the more mature intestinal characteristics necessary to supply the protective factor to the infant rat recipients.

Based on our previous studies, indicating that bacterial flora induced protection against *C. parvum* infection in mice, it was possible that transferring the mucosal preparations from adult animals introduced bacterial species into the infant intestine that conferred resistance to *C. parvum* infection. To determine if the protective activity in the adult rat mucosa was a result of introducing adult microflora, the mucosal preparation was subjected to irradiation, sufficient to kill any live bacteria. Following irradiation, no bacteria were found on aerobic and anaerobic cultures of the preparation. The irradiated preparation was then fed to infant rats and found to reduce infection with *C. parvum* to a similar degree as seen in the group receiving non-irradiated adult rat intestinal scrapings (Akili and Harp, 2000). These data indicate that live bacteria were not required for the protective effect of the preparation. It is still possible that the effects may be mediated by a bacterial product not affected by the irradiation process.

Boiling the intestinal preparation resulted in a loss of protective activity. The percentage of infected animals in the group receiving boiled scrapings was similar to that seen in the control group (Akili and Harp, 2000). This indicated that the substance responsible for the activity in the preparation was heat labile. There are a number of bacterial toxins that are heat labile, as well as several that are heat stable (Guerrant *et al.*, 1999). In addition, a myriad of host cellular components may be present in the scrapings preparations, some of which would be heat labile, including most proteins.

We also tested mucosal preparations from adult cows for effectiveness in protecting infant mice from *C. parvum* infection. We dosed infant mice with mucosal preparations twice daily from 5 to 9 days of age, and challenged them with *C. parvum* at 7 days of age. Control mice were not treated with mucosal preparation, but were similarly challenged with *C. parvum* at 7 days of age. At 14 days of age all mice were killed and examined for infection. Ten of 11 control mice were infected with *C. parvum*, while only 1 of 7 treated mice was infected (Akili and Harp, unpublished). In sum, these data indicate that the intestinal mucosa of adult animals (at least rats and cows) resistant to *C. parvum* contains a substance that, when fed to susceptible infant rats and mice, can transfer protection against *C. parvum* infection.

Changes in intestinal epithelium following *C. parvum* infection

In mice and other rodents, expression of intestinal enzymes is a developmentally regulated event (Quaroni, 1985; Henning, 1987). Before weaning, lactase is expressed on the brush borders of epithelial cells, and sucrase is essentially absent. Around the time of weaning (about 3 weeks of age in mice), sucrase increases, and lactase decreases to low levels. We compared the expression of these two enzymes in normal mice, and mice infected with *C. parvum* at 1 week of age, using a

standard colorimetric assay (Harp *et al.*, 1999). In normal mice, sucrase was essentially absent at 1 week, 10 days and 2 weeks of age, and then rose sharply at 3 weeks. Lactase was elevated at 1 week, 10 days and 2 weeks, and then dropped to low levels at 3 weeks. In contrast, in mice infected with *C. parvum* at 1 week of age, sucrase increased at 2 weeks, and lactase decreased to low levels at 2 weeks (Fig. 2). These data suggested that infection with *C. parvum* accelerated the onset of mature epithelial-cell phenotype, i.e. increased expression of sucrase and decreased expression of lactase.

A further measure of epithelial cell maturation is rate of proliferation. In rodents, epithelial cell proliferation is low in the first 2 weeks of life, and then increases dramatically beginning in the third week after birth (Klein, 1989). We compared proliferation by crypt epithelial cells in normal and *C. parvum*-infected mice. In both groups, at 1 week and 10 days of age, 25–50% of the cells in the crypt zone were found to be proliferating. At 2 weeks of age, 50% of the cells in normal mice were proliferating; in contrast, in *C. parvum*-infected mice, nearly all cells were proliferating (Harp *et al.*, 1999). Thus it appears that proliferation of intestinal epithelial cells is increased in mice following infection with *C. parvum*.

The changes seen in expression of sucrase and lactase could be related to the increased epithelial cell proliferation seen in mice infected with *C. parvum*. Intestinal epithelial cells may be programmed to begin expressing more sucrase and less lactase once a certain number of cell divisions have occurred. The increased proliferation of epithelial cells in mice infected with *C. parvum* would result in the critical number of divisions being reached sooner (2 weeks of age rather than 3 weeks) and in accelerated expression of the 'mature' phenotype with respect to enzyme expression.

Changes in the phenotype of intestinal epithelial cells, either following *C. parvum* infection or as a result of normal maturation, might be relevant to increased resistance to infection. It has been reported that *C. parvum* infection of the intestinal epithelium may involve interaction of a lectin-like receptor with simple sugars or complex carbohydrates (Kuhls *et al.*, 1991; Thea *et al.*, 1992; Llovo *et al.*, 1993). One might hypothesize that expression of sucrase may be regulated coordinately with expression of a lectin-like receptor that is critical for *C. parvum* infection of intestinal epithelium. For example, expression of sucrase may be accompanied by downregulation of such a receptor, or may sterically hinder interaction of that receptor with the parasite. Interestingly, we found that feeding sucrose to infant mice increased resistance to *C. parvum* infection (Harp, 1999).

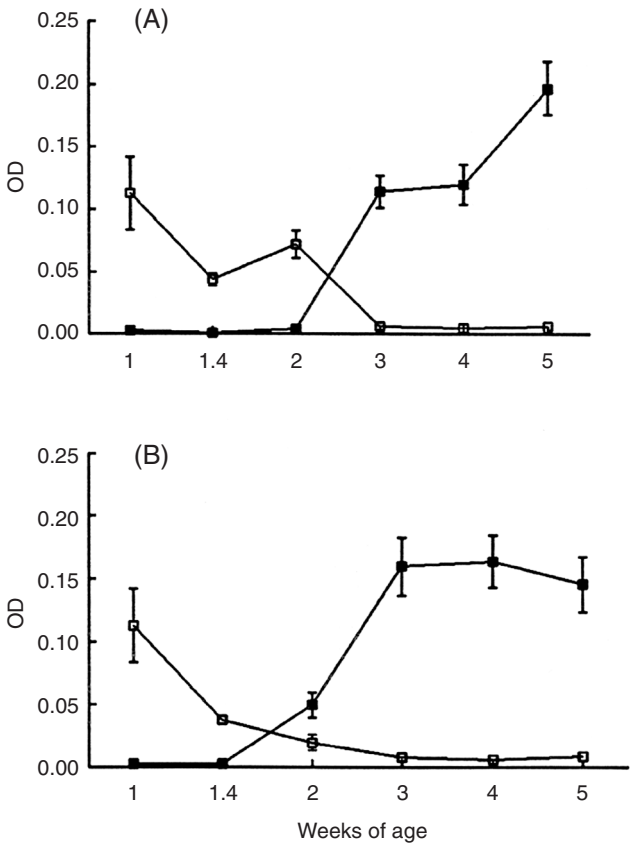

Fig. 2. Levels of sucrase [■] and lactase [□] in ileal tissues of (A) normal mice, and (B) mice infected with *C. parvum* at 1 week of age. Data are presented as mean optical density reading ± SEM, minimum of 10 mice per group. (Reprinted with permission from Harp *et al.*, *Journal of Eukaryotic Microbiology* **46**: 64–65S, 1999.)

Conclusions

It is clear that there is still much to be learned about the complex interactions between innate and specific immune response, physiological maturation, and the role of intestinal flora in mediating resistance to *C. parvum* infection. It seems to be well established that both $CD4^+$ cells and IFN-γ are important for resistance to, and recovery from, *C. parvum* infection in mice (reviewed by Theodos, 1998; Riggs, 2002). Studies in both murine and bovine systems suggest a role for $CD8^+$ intraepithelial lymphocytes as well (Abrahamsen *et al.*, 1997; Wyatt *et al.*, 1997: reviewed by Riggs, 1997). Studies outlined in this review suggest that at least one additional factor critical to resistance to *C. parvum* is (or is triggered by) the presence of intestinal microflora. In addition to studies in mice, it is interesting to note that development of resistance to *C. parvum* in bovine calves may be correlated with the development of intestinal and rumenal microflora (Harp *et al.*, 1990). One may further speculate that, in addition to the deficit of $CD4^+$ lymphocytes seen in AIDS patients, poor nutrition and antibiotic therapy might, in some cases, result in altered microflora in these individuals, which could then contribute to increased susceptibility to *C. parvum* infec-

tion. Preliminary studies indicate that treatment of *C. parvum*-infected AIDS patients with Lb may be of benefit in alleviating symptoms (De Simone *et al.*, 1995). The presence of a substance in intestinal mucosa of adult animals resistant to *C. parvum* that can transfer resistance to susceptible infants supports the hypothesis that maturational changes in the intestine also contribute to host resistance to infection. That *C. parvum* itself can alter developmentally regulated expression of intestinal enzymes is also an intriguing finding, and may be relevant to the apparent window of susceptibility in species, such as bovine calves, that mature in an environment often heavily contaminated with the parasite.

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